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AUTHOR(S):

Azuma, Jun-ichi; Sakanaka, Makoto; Ming, Zhang; Okamura, Keizo

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Preparation of Manno-oligosaccharides by Acetolysis of Mannan

Jun-ichi AZUMA, Makoto SAKANAKA*, Zhang MING and Keizo OKAMURA

加酢分解によるマンノオリゴ糖の調製

東　順一・阪中　誠*・張　鳴・岡村　圭造

Abstract

A new preparation method of homologous series of β-(1→4)-linked D-manno-oligosaccharides was developed. Manno-oligosaccharide acetates were prepared by acetolysis of mannan isolated from ivory nut meal by extraction with 7% potassium hydroxide and saponified to give a mixture of manno-oligosaccharides. The condition of hydrolysis for 45 min at 50°C was found to be an optimum for the preparation of manno-oligosaccharides. By a size exclusion chromatography on Toyopearl (Fractogel) HW40S, manno-oligosaccharides having degree of polymerization up to 8 were isolated and characterized by NMR spectroscopic analysis.

要　旨

β-(1→4) 結合した D-マンノオリゴ糖の調製法を開発した。ゾウゲヤシより7%の水酸化カリウムを用いて抽出したマンナンを種々の条件下で加酢分解した後ケン化したところ，マンノオリゴ糖の調製には50°C，45分の反応が最適であることがわかった。得られたマンノオリゴ糖について Toyopearl (Fractogel) HW40S を用いた立体排除クロマトグラフィーを行った結果，8量体までのマンノオリゴ糖を単離することができた。また，得られたマンノオリゴ糖及びマンナンの性質を NMR により解析した。

1. Introduction

A series of β-(1→4)-linked D-manno-oligosaccharides have been isolated from partial acid and enzymatic hydrolyzates of ivory nut mannan[1-5], copra meal[6] and mannan[7], legume seed galactomannans[8,9], mucilages[10,11], Konjac glucomannan[12], and wood gluco-

* 浜理薬品工業株式会社 (Hamari Chemicals, Ltd.)

mannans[13-19]. These manno-oligosaccharides were expected to give unambiguous proof for the chemical structure of the original polysaccharides. However, no systematic work has been reported on this subject until now.

In the course of investigating the effects of CO_2 laser on hemicellulose, especially on glucomannan, these manno-oligosaccharides were required as the authentic standard and the model compounds. Previously, we developed a convenient preparation method of cello-oligosaccharides by partial hydrolysis of Whatman CF-11 cellulose powder with 72% sulfuric acid followed by HPLC[20]. At first, we tried to apply this method for preparation of manno-oligosaccharides. However, the concentrated sulfuric acid was found to be inadequate for partial hydrolysis of ivory nut mannan because of its lower molecular weight. Instead, acetolysis was found to give a mixture of manno-oligosaccharides in a high yield.

In this study, we present an extensive study on the preparation of manno-oligosaccharides by acetolysis of ivory nut mannan.

2. Experimental

2.1 General

Optical rotations were determined with a JASCO DIP-181 digital polarimeter at 25°C. The values of molar optical rotation [M] were calculated from those of specific optical rotation, $[\alpha]_D$.

Thin layer chromatography (TLC) was carried out on Kieselgel 60 plates (0.5 mm, Art. 5744, Merck) with (A) 1-butanol-2-propanol-water (3 : 12 : 4, v/v) and (B) 1-butanol-ethanol-water (5 : 3 : 2, v/v) as irrigants. Spots were detected by spraying with 10% sulfuric acid and charring by heating.

Gas liquid chromatography (GLC) was carried out[21] on a Shimadzu GC-7AG gas chromatograph equipped with flame ionization detectors. Separation was performed on 3% ECNSS-M on Gas Chrom Q in a glass column (2 m×0.3 cm) at 190°C.

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-200 NMR spectrometer (200 MHz for ^1H and 50.3 MHz for ^{13}C) in deuterium oxide. The ^1H-NMR spectra were obtained at 90°C and chemical shifts in p.p.m. for anomeric protons were given with sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl) propionate (TSP) as an internal standard. The ^{13}C-NMR spectra were obtained at 70°C with complete proton-decoupling and with gated decoupling, and chemical shifts in p.p.m. were given with 1,4-dioxane (67.40 p.p.m.) as an internal standard.

High performance liquid chromatography (HPLC) was carried out on a system using a JASCO 880-PU intelligent HPLC pump fitted with Reodyne 7125 sample injection valve, Kyoto Chromato CH-250 column oven, and Shodex RI SE-51 differential refractometer. The prepacked polyvinyl alcohol gel columns (7.6 mm×50 cm) of Asahipak GS-220 and GS-320 were used at 60°C and at a flow rate of 0.6 ml/min. Distilled water was used as an eluent. The pressure of the columns was in the range from 1.4 to 1.8 MPa. Chromatograms were recorded and integrated with a Waters M740 data Module.

2. 2 Preparation of ivory nut mannan

Ivory nut (*Phytelephas macrocarpa*) were peeled by an electric grinder, crushed and milled to pass 60 mesh screen. The ivory nut meal was extracted with ethanol-benzene (1 : 2, v/v) for 24hr. The extractive-free ivory nut meal was extracted thrice with 20 vol. of 7% potassium hydroxide at room temperature for 24 hr under nitrogen atmosphere[22]. Each extract was recovered by filtration through 15G1 sintered glass filter, neutralized with acetic acid, dialyzed against distilled water, concentrated to a small volume, and poured into 5 vol. of ethanol. The precipitated material was recovered by centrifugation, washed with acetone followed by petroleum ether to give mannan in 17% yield.

2. 3 Acetolysis of mannan

Five grams of the dried ivory nut mannan was added with vigorous stirring to mixtures of glacial acetic acid (19 ml), acetic acid anhydride (19 ml), and sulfuric acid (2 ml) kept at four different temperatures (30°C, 40°C, 50°C, and 60°C). Five milliliters of the partial hydrolyzate was taken out at various time intervals and poured into ice-water. The acetolyzate was neutralized with sodium carbonate and filtered to obtain a mixture of manno-oligosaccharide acetates. The manno-oligosaccharide acetates were solubilized in 5 ml of dichloromethane and deacetylated by treatment with potassium hydroxide (0. 5 g) in 8 ml of a toluene-methanol mixture (1 : 3, v/v) for 1 hr at room temperature. The water-soluble manno-oligosaccharides were recovered by repeated addition of distilled water and centrifugation followed by deionization with Dowex 50-X8 (H^+ form) and 1-X8 (acetate form) resins. Composition of manno-oligosaccharides was analyzed by size exclusion chromatography on Asahipack GS-220.

2. 4 Separation and analysis of manno-oligosaccharides

About 500 mg of the manno-oligosaccharide mixture was solubilized in a few ml of distilled water and applied on a column (6 cm×112 cm) of Toyopearl (Fractogel TSK) HW40S (TOSOH) and eluted with distilled water at a flow rate of 5. 0 ml/min and a pressure of 0. 9-1. 0 MPa (Knauer HPLC 64 pump). The elution was monitored by Model R401 refractometer (Waters). The individual manno-oligosaccharides were collected automatically with Model SF-139 peak collector (Advantec Toyo) actuated at 220 drops (9. 4 ml) per one fraction, and evaporated to dryness. The isolated manno-oligosaccharides were purified by repeated chromatography on the same column. Purity of the isolated oligosaccharides was checked by size exclusion chromatography on Asahipak GS-220.

2. 5 Determination of degree of polymerization

Degree of polymerization (D. P., n) of the isolated manno-oligosaccharides was determined from ^{1}H-NMR spectroscopic analysis and the plots of $R_f/(1-R_f)$ against D. P.[23] and $[M]_n/n$ against $(n-1)/n$[24].

3. Results and Discussion

3.1 Characterization of ivory nut mannan

The isolated mannan corresponded to mannan A previously isolated by Aspinall *et al*[22]. and had $[\alpha]_D^{25}$ value of $-40.7°$ (*c*, 1.1 in N NaOH). Acid hydrolysis followed by GLC as alditol acetates yielded arabinose (2.5%), mannose (92.3%), galactose (4.5%), and glucose (0.6%). Although ivory nut mannan A which is extractable with 7% potassium hydroxide has been reported to be less soluble in water[1], 93.5% of the mannan isolated in this paper was found to be soluble in water, indicating its lower molecular weight. The same observation has been reported by Thiem *et al*[2]. They analyzed the molecular weight distribution of mannan A by gel filtration on Sephadex G-25 and found its molecular weight to be in the range of 830 to 4,100 corresponding to D.P. from 5 to 25. Aspinall *et al*[22]. also showed by methylation analysis that mannan A was composed of an average of 10 to 13 residues. In our study, the weight average molecular weight of the water-soluble fraction of the isolated mannan was estimated to be 1,600 corresponding to D.P. about 10 by size exclusion chromatography on Asahipak GS-320 using the isolated manno-oligosaccharides and dextrans having known molecular weights (Pharmacia) as the calibration standard. The same D.P. value was also obtained by ^{1}H-NMR spectroscopic analysis by integrating the anomeric protons due to non-reducing end (H-1n), intermediate (H-1i) and reducing end (H-1α and H-1β) mannose residues (Fig. 1, Table 1). All anomeric proton signals had $J_{1,2}$ close to 0.9 Hz, compatible with the expected $^4C_1$ conformation of the D-mannopyranose residues. Presence of appreciable proportion of non-reducing and reducing end mannopyranose residues was also confirmed by ^{13}C-NMR measurement (Fig. 2, Table 2). The ^{13}C-NMR spectrum of the present mannan could be assigned based on the data recorded in 5% NaOD-D_2O[25]. From the value (160.4 Hz) of one-bond ^{13}C-^{1}H coupling constant, the anomeric configuration of the non-reducing and intermediate glycosidic linkages of D-mannopyranose residues was β. Similarly, the signals due to the reducing end D-mannopyranose residue appeared at 94.7 p.p.m. and 94.6

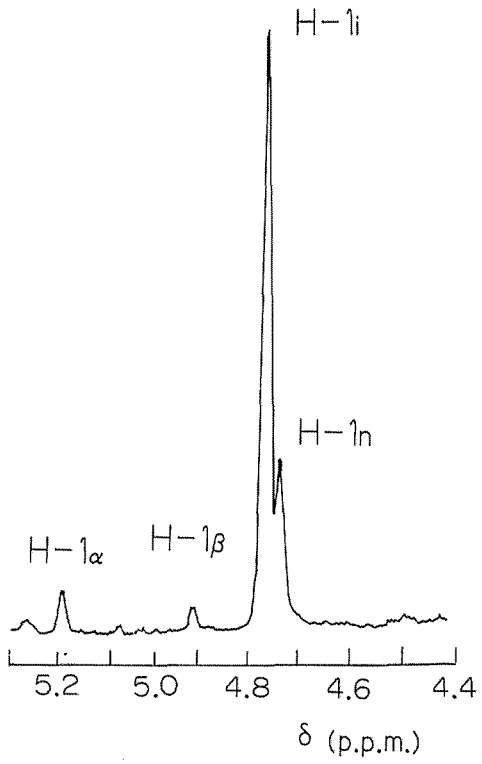

Fig. 1 ^{1}H-NMR spectrum of ivory nut mannan at anomeric region in deuterium oxide at 90°C: (H-1α, H-1β), H-1i, and H-1n represent anomeric protons of the reducing end, intermediate, and non-reducing end residues. Chemical shifts of these protons were assigned as listed in Table 1.

Table 1 Properties and ^{1}H-NMR data for β-(1→4)linked D-manno-oligosaccharides

Oligomers	Melting points(°C)		$[\alpha]_D^{25}$		^{1}H-NMR data (p. p. m.)			
	Obs.	Lit.[1)-19)]	Obs.	Lit.[1)-19)]	H-1α	H-1β	H-1i	H-1n
Mannobiose	189—191	198—201	−8.9° (H_2O)	−7° ～−9° (H_2O)	5.19 (1.5)[a] [0.5][b]	4.90 (1.1) [0.5]		4.73 (1.0) [1.0]
Mannotriose	166—168 ($3H_2O$)	134—167($3H_2O$) 219 (anhyd.)	−23.4° (H_2O)	−20° ～−25° (H_2O)	5.19 (1.6) [0.5]	4.90 (1.1) [0.5]	4.76 (1.1) [1.0]	4.72 (0.9) [1.0]
Mannotetraose	226—228	228—232	−28.7° (H_2O)	−29° ～−31° (H_2O)	5.19 (1.5) [0.5]	4.90 (1.1) [0.5]	4.75 (1.1) [2.1]	4.72 (1.0) [1.0]
Mannopentaose	—	—	−30.8° (H_2O)	−30.2° (H_2O)	5.19 (1.5) [0.6]	4.90 (1.1) [0.4]	4.75 (1.0) [3.1]	4.73 (1.0) [1.0]
Mannohexaose	—	—	−33.2° (H_2O)	—	5.19 (1.5) [0.6]	4.90 (1.1) [0.4]	4.75 (1.0) [3.8]	4.72 (1.0) [1.0]
Mannoheptaose	—	—	−35.7° (H_2O)	—	5.19 (1.4)[a] [0.6][b]	4.90 (1.1) [0.4]	4.75 (1.1) [5.2]	4.72 (1.1) [1.0]
Mannooctaose	—	—	−37.2° (H_2O)	—	5.19 (1.5) [0.6]	4.90 (1.1) [0.4]	4.75 (1.1) [5.8]	4.73 (0.9) [0.7]
Mannan	—	—	−40.7° (N NaOH)	−46° (N NaOH)	5.19 (1.4) [0.5]	4.91 (1.1) [0.3]	4.76 (0.9) [8.4]	4.73 (0.9) [1.0]

[a] Coupling constant ($J_{1,2}$, Hz). [b] Molar ratio.

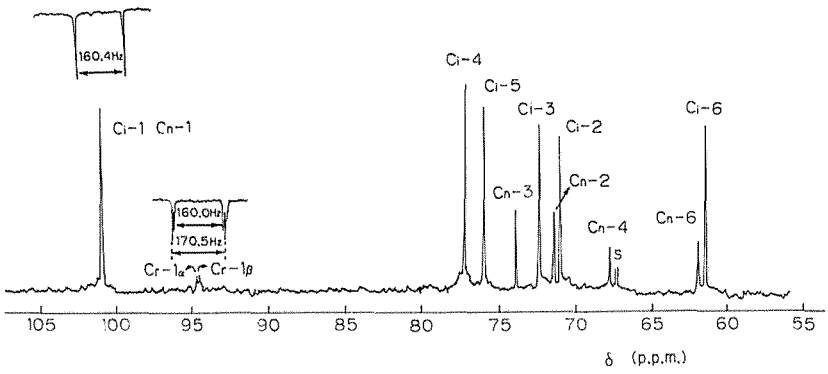

Fig. 2 ^{13}C-NMR spectrum of ivory nut mannan in deuterium oxide at 70°C. Inserted spectra were obtained with gated decoupling to determine anomeric $^1J_{C,H}$ coupling constants (Hz). Symbols: dioxane (s), non-reducing end residue (C_n), intermediate residues (C_i), and reducing end residue (C_r).

Table 2 ^{13}C-NMR data for β-(1→4)-linked D-manno-oligosaccharides and ivory nut mannan (chemical shifts, in p. p. m.[a])

Residues		Oligomers							
		Mannobiose		Mannotriose		Mannotetraose		Mannopentaose	
		α	β	α	β	α	β	α	β
Reducing end residue	C-1	94.69	94.56	94.68	94.68	94.71	94.59	94.70	94.55
	C-2	71.27	71.58	71.31	71.61	71.34	71.64	71.34	71.64
	C-3	69.85	72.51	69.83	72.54	69.86	72.58	69.85	72.53
	C-4	77.70	77.38	77.65	77.33	77.69	77.35	77.69	77.35
	C-5	71.91	75.69	71.90	75.67	71.94	75.72	71.94	75.70
	C-6	61.59	61.59	61.53	61.51	61.51	61.51	61.51	61.51
Internal residue	C-1			100.92		101.00		100.99	
	C-2			70.90		70.96, 70.90		70.94	
	C-3			72.39		73.41		72.39	
	C-4			77.35		77.35		77.35	
	C-5			75.91		75.95		75.95	
	C-6			61.53	61.51			61.51	
Non-reducing end residue	C-1	100.99		101.01		101.00		100.99	
	C-2	71.34		71.37		71.40		71.38	
	C-3	73.86		73.84		73.87		74.84	
	C-4	67.71		67.71		67.71		67.71	
	C-5	77.24		77.24		77.35		77.35	
	C-6	61.95		61.95		61.95		61.94	

Residues		Oligomers							
		Mannohexaose		Mannoheptaose		Mannooctaose		Mannan	
		α	β	α	β	α	β	α	β
Reducing end residue	C-1	94.70	94.56	94.68	94.56	94.71	94.59	94.70	94.55
	C-2	71.31	71.64	71.31		71.31		[170.5]	[160.0][b]
	C-3	69.85	72.54	69.85	72.54	69.85			
	C-4	77.65	77.35	77.65		77.65			
	C-5	71.93	75.69	71.93		71.93			
	C-6	61.51	61.51	61.51		61.51			
Internal residue	C-1	100.99		100.99		100.98		100.98	[160.4]
	C-2	70.94		70.94		70.95		70.95	
	C-3	72.38		72.39		72.39		72.37	
	C-4	77.33		77.30		77.29		77.29	
	C-5	75.95		77.96		77.95		95.97	
	C-6	61.51		61.51		61.50		61.48	
Non-reducing end residue	C-1	100.99		100.99		100.98		100.98	
	C-2	71.38		71.38		71.38		71.39	
	C-3	73.83		73.83		73.83		73.83	
	C-4	67.71		67.71		67.70		67.70	
	C-5	77.25		67.30		67.30		77.29	
	C-6	61.95		61.94		61.94		61.94	

a) In p. p. m. relative to internal 1, 4-dioxane (67.40 p. p. m. from TMS).
b) Coupling constant ($J_{C,H}$, Hz).

p. p. m. were assigned to be α- and β-D-mannopyranoses from their coupling constants, 170. 5 Hz and 160. 0 Hz, respectively. Based on these physicochemical analyses, we concluded that the molecular weight of the ivory nut mannan extractable with 7% potassium hydroxide is low.

3. 2 Preparation of manno-oligosaccharide mixture by acetolysis

Time course and temperature dependence of the conversion of the mannan to water-soluble oligosaccharides were analyzed by acetolysis followed by saponification. Figure 3 shows the change of conversion rate within 3 hr at four different acetolysis temperatures (30°C, 40°C, 50°C and 60°C). Raising the hydrolysis temperature resulted in substantial increase of the conversion rate. In contrast, the conversion rate initially increased with hydrolysis time but became lower by hydrolysis after 2 hr.

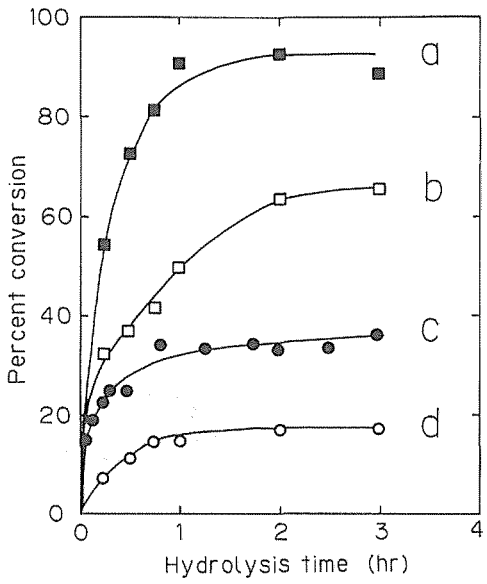

Fig. 3 Conversion rate of ivory nut mannan into manno-oligosaccharides by acetolysis followed by saponification. The amount of oligosaccharides soluble in water at acetolysis at (a) 60°C (■), (b) 50 °C (□), (c) 40°C (●), and (d) 30°C (□).

3. 3 Effects of hydrolysis temperature and time on composition of manno-oligosaccharides

Figure 4 shows the effects of hydrolysis time and temperature on composition of manno-oligosaccharides. The proportion of mannose increased with hydrolysis temperature and time and attained to 93% after 3 hr at 60°C. The proportion of mannobiose increased similarly to that of mannose up to 50°C, but a hydrolysis time longer than 1. 0 hr at 60°C promoted further hydrolysis to mannose. At 30°C and 40°C, a homologous series of manno-oligosaccharides having D. P. 3-8 could well be detected within 2. 0 hr. At 50°C, the formation of the same oligosaccharides reached a maximum at 45 min and rapidly decreased by prolonged hydrolysis. At 60°C, no mannoheptaose and mannooctaose could be detected and the production of the other oligosaccharides having D. P. 3-6 attained maximum after 30 min. Based on these results together with those shown in Fig. 1, the acetolysis for 45 min at 50°C was concluded to be optimal for production of manno-oligosaccharides.

3. 4 Separation of manno-oligosaccharides

About 500 mg of the manno-oligosaccharide mixture prepared by acetolysis of ivory nut mannan at 50°C for 45 min followed by saponification was applied on a column of Toyopearl HW40S. Figure 5 shows a typical elution profile which indicates that the manno-oligosaccharides having D. P. of 2-8 were separated within 4 hr. By a single

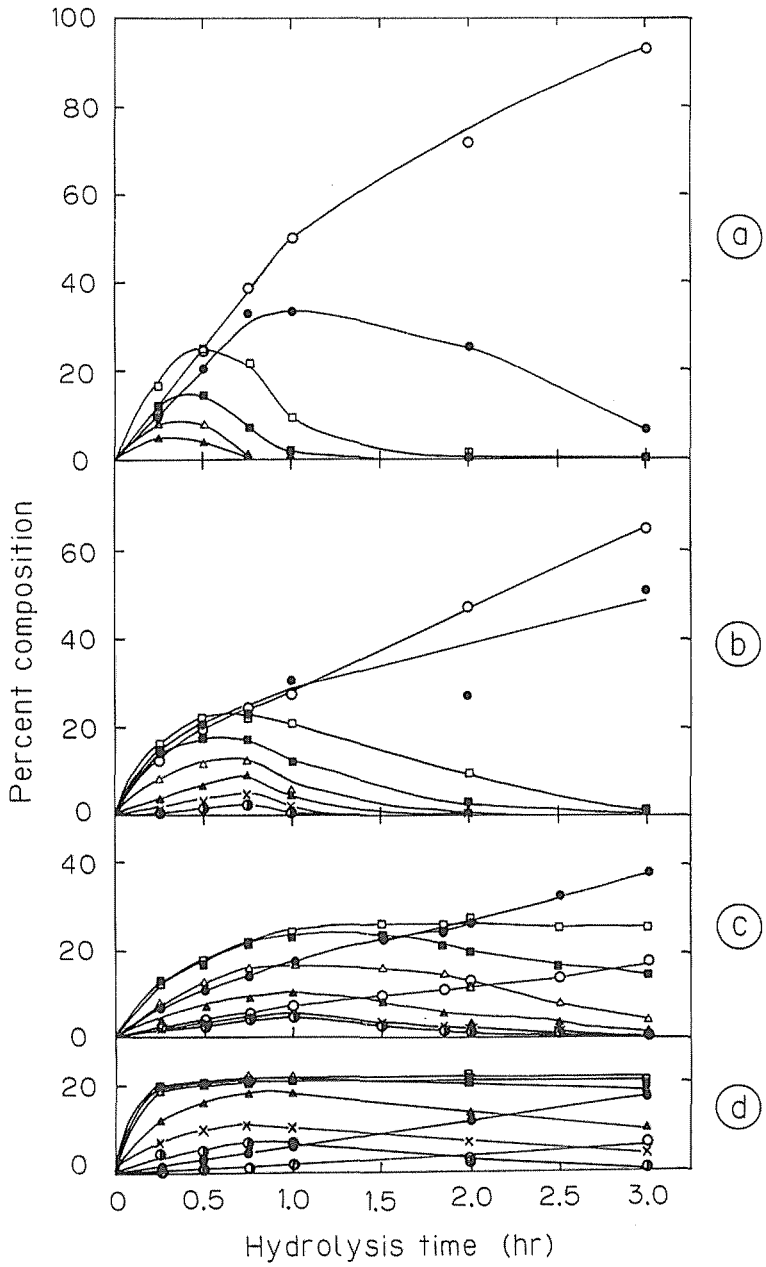

Fig. 4 Effects of acetolysis time and temperature on oligosaccharide distribution profile: mannose (○), mannobiose (●), mannotriose (□), mannotetraose (■), mannopentaose (△), mannohexaose ((▲), mannoheptaose (×), and mannooctaose (◑). Acetolysis temperatures at (a) 60°C, (b) 50°C, (c) 40°C, and (d) 30°C.

chromatography, 102 mg of mannobiose, 90 mg of mannotriose, 60 mg of mannotetraose, 41 mg of mannopentaose, 22 mg of mannohexaose, 9.8 mg of mannoheptaose, and 4.5 mg of mannooctaose were separated in 84% recovery. Each oligosaccharide was purified by repeated rechromatography on the same column. The properties of the isolated oligo-

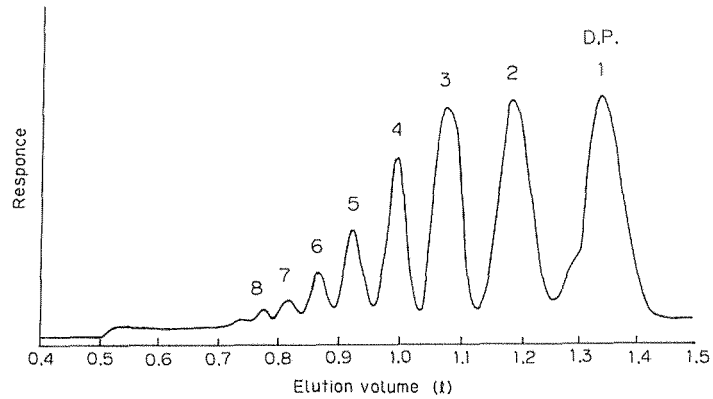

Fig. 5 Elution profile of manno-oligosaccharides on Toyopeal HW40S. The numbers (1 to 8) represent the degree of polymerization.

saccharides are summarized in Table 1. Previously Toyopearl HW40S gel was applied to separate homologous series of malto- and isomalto-oligosaccharides[26,27]. The present study indicates that this gel can also be applicable for the separation of homologous series of manno-oligosaccharides.

The structure of the isolated manno-oligosaccharides was analyzed by ^{1}H- and ^{13}C-NMR measurements. The assignments of the signals due to manno-oligosaccharides toge-

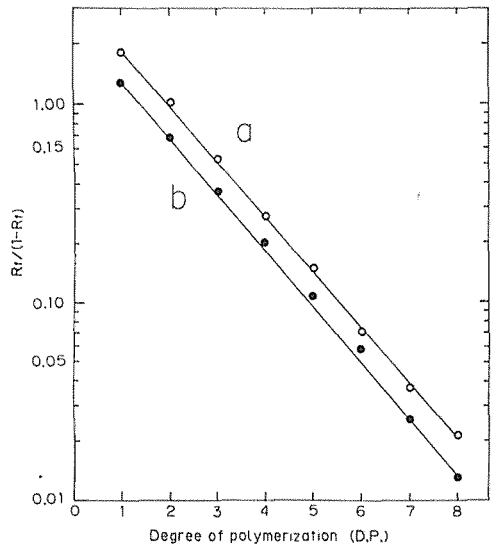

Fig. 6 Relation between $R_f / (1-R_f)$ and degree of polymerization of manno-oligosaccharides: The R_f values were obtained by TLC using (a) 1-butanol-2-propanol-water (3 : 12 : 4, v/v) (○) and (b) 1-butanol-2-ethanol-water (5 : 3 : 2, v/v) (●) as irrigants.

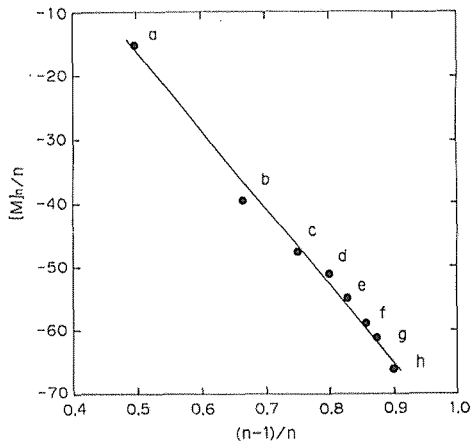

Fig. 7 Relation between $[M]_n/n$ and $(n-1)/n$, where [M] and n were molar optical rotaion and degree of polymerization, respectively : mannobiose (a), mannotriose (b), mannotetraose (c), mannopentaose (d), mannohexaose (e), mannoheptaose (f), mannooctaose (g), and ivory nut mannan (h).

ther with original mannan were listed in Tables 1 and 2. Previously, ^{13}C-NMR signals of mannobiose and mannotriose have been assigned by Usui *et al*[28]. and McCleary *et al*[9]. In this study, we extended the assignment of signals up to mannooctaose. With increase of D. P. the intensity of the 6 carbon signals becomes strong due to the intermediate mannopyranosyl residues and the spectrum of mannooctaose becomes similar to that of the mannan. In the case of ^{1}H-NMR, the anomeric protons of the reducing end, intermediate and non-reducing end mannopyranose residues appeared at 4.90 p. p. m. (H-1β) and 5.19 p. p. m. (H-1α), 4.75 p. p. m. (H-1i) and 4.72 p. p. m. (H-1n), respectively, as doublets ($J_{1,2}$ 0.9-1.0 Hz). Accordingly, all the D-mannopyranose residues were deduced to be in the $^{4}C_{1}$ conformation. The D. P. values of the isolated manno-oligosaccharides were determined by integrating these signals and the results are also listed in Table 1. As the D. P. of the manno-oligosaccharides became higher, the signal intensity due to the intermediate mannose residues predominated as observed in their ^{13}C-NMR spectra. The D. P. values of the isolated manno-oligosaccharides were also determined by TLC and optical rotation measurement. Figure 6 shows plots of $R_f/(1-R_f)$ against D. P. using two different irrigants. Both plots showed a good linear relationship, indicating that the isolated manno-oligosaccharides were homologous. Secondly, molecular rotation values of the isolated manno-oligosaccharides and the original mannan were calculated and $[M]_n/n$ values were plotted against $(n-1)/n$, where n equals D. P. Figure 7 showed again a good linear relationship, confirming the results that the isolated manno-oligosaccharides together with the original mannan were homologous.

In summary, the present results indicate that the acetolysis followed by saponification and chromatography on Toyopearl HW40S provide an effective preparation method of standard manno-oligosaccharides.

Acknowledgement

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